BIOLOGICAL AGENT DETECTION IN FOOD WITH AN ARRAY BIOSENSOR

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ABSTRACT

Intentional contamination of food with biological agents is a global concern. Most identification methods for these agents in food require extensive pre-treatment or concentration prior to analysis in a laboratory and take up to 4 days for bacterial agents. The Array Biosensor was developed for simultaneous analysis of multiple samples for multiple agents in a portable format. The simultaneous analysis of more than 6 agents takes less than 20 minutes. Analysis results of various foods spiked with SEB, *E. coli* O157:H7, *Salmonella*, and other bacterial agents will be presented.

INTRODUCTION

The intentional contamination of our food and water supply by terrorists is a major area of concern. Two cases in the U.S. have been documented.[1] In 1984, salad bars in Oregon were intentionally contaminated with *Salmonella* typhimurium by members of a religious commune attempting to influence a local election. Two years later at a large medical center in Texas, pastries tainted with *Shigella* dysentariae were left in a staff break room; twelve laboratory workers became infected. Food and water are good targets in that the biological agent unnoticed might be ingested by the warfighter, homeland defender or civilian, thereby causing illness or death. If there was no proclamation by the terrorists or other group that biological agents had been released, there would be a delay in identifying an incident due to the nondescript initial symptoms. Only with a significant number of people falling ill would terrorism be suspected. If a message is received about a bioterrorist attack, the first responders would need to identify the agent rapidly. The test would need to be easy to use, detect for more than one agent in a limited number of samples and be low in cost.

Maslanka describe three levels of public health response requirements.[1] The first is for surveillance, which has a low requirement for speed, low cost constraints, high strain discrimination, and moderate ease of use. The next level is in response to an outbreak and this level requires high speed, medium cost, and strain discrimination only if needed for treatment and moderate ease of use. For a bioterrorist event

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(third level), the requirements are rapid detection, high cost constraints, and strain discrimination only if needed for treatment and high ease of use.

Traditional methods of detection of bacterial agents involve growing of the bacteria in an pre-enrichment broth for 1-4 days, then growing for 6 or more hours in an enrichment broth, followed by colony formation on agar plates and examination of the colonies that grow. Recently, Peng and Shelef developed a 24-hour assay dependent on the growth stage for *Salmonella* and *Listeria*.[2] As the cells increase, the media turns blackish and a decrease in light transmission is observed. This is very time consuming and labor intensive. Many of the bacterial agents could cause serious, if not lethal, medical problems before the analysis is completed. Recently, work has been done on improving the analysis of the enriched sample using DNA and antibodies.

DNA analysis includes polymerase chain reactions (PCR) and/or hybridization using microarrays.[3-5] This technique can be very sensitive but the need to grow up the cells for the bacterial agents in some sort of enrichment broth for 6 to 48 hours is problematic. Even if an enrichment step is performed, but the agent of interest is at low concentrations versus other cells, it is hard to identify the infectious agent. Immunomagnetic separation (IMS) has proven to be the most successful technique for separating the cells of interest from the enrichment media prior to PCR or hybridization. In IMS, magnetic beads are coated with antibodies against the bacteria of interest. The beads are then separated from the media by magnet. Another concern is that DNA-based techniques only identify *actively growing* cells. In some cases, the bacterial cells of interest could have been stressed, thereby affecting their growing ability. A few groups have developed PCR techniques which identify more than one agent;[6,7] this includes genotypes of a specific organism.[9] While these techniques have detection limits as low as 10 CFU/ml, their forte might be more in the area of confirmation and genotyping.

Antibody-based assays have been developed which can be used after cell enrichment or without any sample pretreatment. The enzyme-linked immunosorbent (ELISA)-based assays that are able to detect 1 to 10 CFU/ml use an enrichment step which generally is not as long as that used for DNA.[9,10] Again, this enrichment step plus the time to perform the ELISA still add up to more than 11 hours. Other ELISAs that are performed without an enrichment step take from 20 minutes to 2.5 hours but at a cost of reduced limits of detection, 10^6-10^7 CFU/ml and 10^4 CFU/ml respectively. ELISA-based assays have been developed for bacteria such as *E. coli, Salmonella, Campylobacter* and *S. aureus*.[11-13] In addition, there are ELISA-based assays for the detection of SEB with detection limits in the high pg/ml to low ng/ml range in less than one hour.[11] Other types of antibody-based detection methods include immunoprecipitation[14], immunoliposomes[15], electrochemiluminescence (i.e., ORIGEN)[16,17], surface plasmon resonance (SPR, BIACORE)[18-20], light addressable potentiometric sensor (LAPS)[21,22], fiber optic biosensor[23-27], and the Array Biosensor. The array biosensor will be discussed below. As with the ELISAs, the most sensitive method (0.05 CFU *E. coli*/g beef) employed an 18-hour enrichment step.[22] In many cases, as the method time decreases, the level of sensitivity also decreases. For the antibody-based assays without an enrichment step, the levels of detection are 10^3 to 10^6 CFU/ml. Current antibody-based methods provide the rapid identification[28] that is needed for an initial response to an outbreak or bioterrorism attack. Longer analysis methods may also be performed for confirmation, but the rapid detection expedites treatment of exposed persons.

The purpose of this work was to adapt a portable, multi-analyte biosensor, referred to as the Array Biosensor, for simultaneous analysis of a minimum of six different biological agents in food in less than 20 minutes. The focus has been the development of five individual assays for the low level detection of Staphylococcal enterotoxin B (SEB), *Escherichia coli* O157, *Salmonella*, *Campylobacter* and *Listeria* spiked into appropriate foods. Analyse of spiked foodstuff appropriate for naturally occurring outbreaks was performed.

BACKGROUND

The Array Biosensor was developed for simultaneous analysis of multiple samples for multiple analytes. The system is composed of three parts: a patterned array of molecular recognition elements, an image capture and data processing system, and an automated fluidics unit. The patterned array of recognition elements, usually antibodies, immobilized on the surface of a planar waveguide is used to capture analyte present in samples. Bound analyte is then quantified by means of fluorescent detector molecules. Upon excitation of the fluorescent label using a small diode laser, a CCD camera detects the pattern of fluorescent antigen:antibody complexes on the sensor surface.

Image analysis software quantitates the fluorescent signals which can be associated with the identity of the analyte. An semi-automated data analysis program[29] is used to calculate the mean fluorescence signal (MFS) values for each square in the matrix. Values three standard deviations above the values for blanks are considered positive.

The array biosensor has been used to identify and quantify toxins, simulants, physiological markers, the MS2 virus and bacteria (gram negative and gram positive). [30-33] Detection of these various agents has been performed in environmental (soil and sand suspensions, clay, pollen and smoke extracts) and clinical matrices (blood, sputum and urine).

METHODS

PREPARATION OF SENSOR SUBSTRATES

Microscope slides are employed as the optical waveguide and sandwich immunoassay were performed on the surface. Rowe *et al.*[30], based on the chemistry of Bhatia *et.al.*[34], have described the preparation of the slides with capture antibody immobilized in a patterned array. First, NeutrAvidin is covalently attached to the slide surface. A poly(dimethylsiloxane) (PDMS) flow chamber is then used to pattern the biotinylated capture antibody in 10 :g/ml in phosphate buffered saline with 0.05% Tween 20, pH 7.4(PBST) in vertical columns on the microscope slide. In some assays biotinylated anti-TNT (10 :g/ml in PBST) was immobilized in columns to provide positive controls. The biotinylated capture antibody was incubated with the NeutraAvidin-coated slide overnight at 4^{o}C. The channels of the flow chamber were washed with PBSTB (PBST with 1 mg/ml bovine serum albumin), the slide removed from the PDMS template and placed in phosphate buffer pH 7.4 containing 10 mg/ml bovine serum albumin. After 30 minutes, the slides were rinsed with deionized water, dried with nitrogen, and stored at 4^{o}C until use.

EXTRACTION PROCEDURE

For meat products (ham and ground beef, 20% fat), 10 g meat and 8 ml of PBSTB were placed into a 37-110 ml mini-sample container on a Waring Blender and blended on high for 2 minutes. The resulting mixture was put into a 50 ml centrifuge tube. The blender container was rinsed with 2 ml PBSTB and the rinse solution was added to the centrifuge tube to yield a 1:1 w/v mixture of meat and buffer. The ham mixture was tested in two formats: spiking the mixture directly or spiking the supernatant after the mixture was centrifuged at 3000 rpm for 5 minutes. The spiked ham and ground beef mixtures were centrifuged at 3000 rpm for 3 minutes to obtain 1 ml supernatant for analysis. The spiked supernatant was used directly. The cantaloupe mixture was prepared in a similar fashion including centrifugation except 5 g of cantaloupe and 5 ml total PBSTB was used to obtain a 1:1 w/v mixture. Carnation non-fat dried milk was prepared per instructions on the box. Into a 15 ml centrifuge tube, 2.7 ml milk and 0.3 ml 10X PBSTB were mixed and analyzed directly. The carcass wash was obtained by adding 100 ml PBS with 1 mg/ml BSA to a 6 lb chicken carcass in a plastic bag. The liquid from the absorbent material in

the chicken wrapping was also added to the bag. The carcass-containing bag was rocked rapidly for 2 hr at room temperature. The liquid "carcass wash" was removed, aliquoted, and frozen until analysis. Eggs were weighed and combined with PBSTB to form a 1:1 w/v mixture. This mixture was then blended for 1 min and used directly for analysis.

ASSAYS

A sandwich immunoassay was performed on the waveguide surface perpendicular to the patterned antibodies. The biotinylated-capture antibody-coated slide was placed into a template containing a PDMS assay mold with the channels running lengthwise on the slide. The slide was hooked up to a Manostat multi-channel pump (Sarah model) with tubing connected to each channel in the PDMS. Syringe barrels (1 ml) were attached to the other end of each slide channel. The PDMS channels were first washed with 1 ml PBSTB. Next, 0.8 ml sample was applied to each channel. After 8 minutes, the sample was removed and the channels washed with 1 ml PBSTB. The fluorescently-labeled tracer antibody (0.4 ml) was applied to the channels. Next, the channels were washed with 1 ml PBSTB, the template disassembled and the slide rinsed in water and dried with nitrogen. The slide was then imaged in the array biosensor.

DATA COLLECTION AND ANALYSIS

The image of the array was captured using SpectraSource software, in the Flexible Image Transport System (FTS) digital format. This intensity image was converted to average intensity per square by a program developed at NRL. This program is described in a Sapsford et al.[35] The average mean intensity was used for analysis.

RESULTS AND DISCUSSION

STAPHYLOCOCCAL ENTEROTOXIN B (SEB) DETECTION ASSAY

The combination of biotinylated IGEN 2B anti-SEB as the capture antibody and AlexaFluor 647 (AF 647) labeled Toxin Technologies sheep anti-SEB antibody proved to be optimum in this study. This combination was employed for the remainder of the study except that the dye to protein (d/p) ratio for the AF 647 sheep anti-SEB was increased to 6.6:1.

An array image of a slide showing the control columns (1 and 6) and the specific columns (2-5) for 0 to 5 ng/ml SEB is shown in Figure 1.

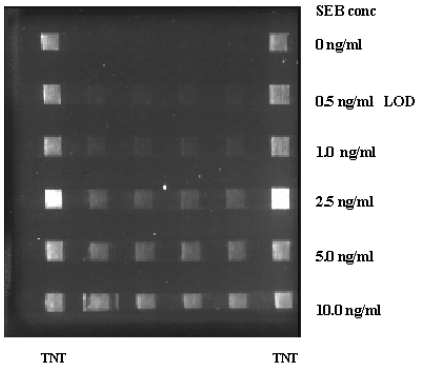


Figure 1: SEB detection in buffer

This image was converted into intensity values for the six squares per row and the six rows. From these values, a SEB standard curve generated in buffer using antibodies described above was determined. Figure 2 shows the results. A minimum of eight mean values was obtained for each concentration. From these results, the limit of detection, lowest concentration that was greater than three standard error of the means (SEM) above the intensity for the blank, was determined to be 0.5 ng/ml. The standard curve fits an asymmetric sigmoid: $y = 1691 + (34485)/(1 + ((x/10.7)^{-2.6}))$. The linear region of the sigmoid is between 0 to 25 ng/ml.

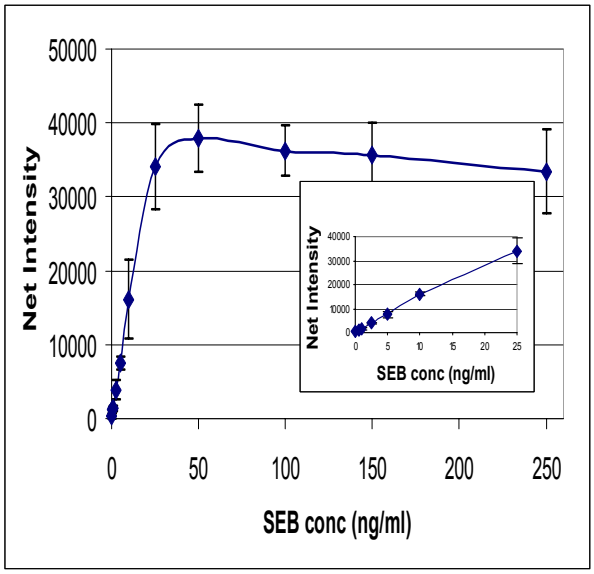


Figure 2: SEB standard curve in buffer. The insert shows the linear region from 0 to 25 ng/ml. Values are an average of 8 or more squares ± SEM.

More important than the standard curve in buffer is how the system works for detection SEB in real food samples. An extraction procedure was developed for several different food matrices. Standard curves for SEB spiked into several different types of food were performed. A representative sample was used; fat and small particulates were not removed. For the food samples with large particulates such as ham, ground beef, and cantaloupe, a centrifugation step was included to remove these large particles to avoid clogging the fluidics.

Figures 3 and 4 show the SEB standard curves determined in meat (ham, ground beef, carcass wash) and non-meat (milk, cantaloupe and egg) respectively. The insets show the curves from 0-25 ng/ml SEB for each food type. The limits of detection and the linear regression results for the curves in the insets are shown in Table 1. There are significant differences in the slopes of the curves for the different matrices including buffer. This would affect quantitation of unknowns if compared to a standard curve in a different matrix. Carcass wash and ham supernatant have the lowest slope and R^2 values less than 0.95, which suggest there is some interference from the matrix which affects binding in the sandwich assay format. Further detailed investigation of the matrix would be needed to determine the exact interferent. In all cases, the detection limit is below the USDA/FSIS solution goal.[36]

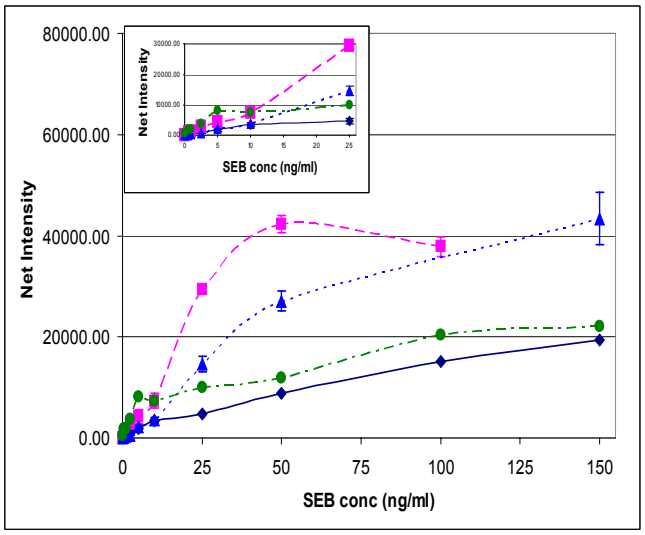


Figure 3: SEB standard curves in meat matrices. Ham supernatant, , solid line; ham extract, ■, dashed line; ground beef, ▲, dotted line; chicken carcass wash, ●, dash-dot line. Insert shows the linear region from 0 to 25 ng/ml. Values are an average of 8 or more squares ± SEM.

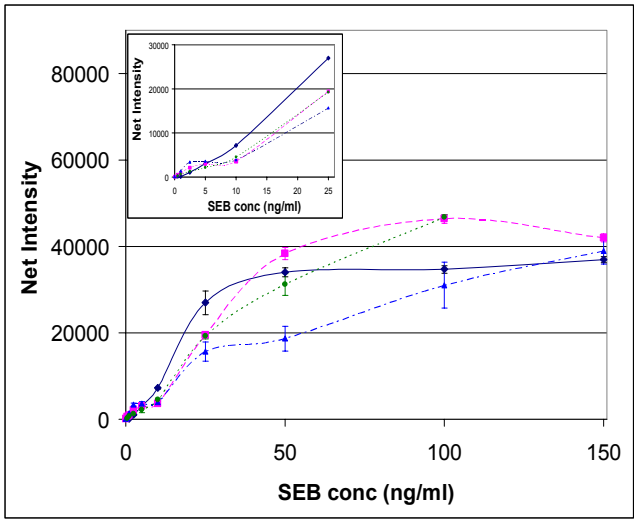


Figure 4: SEB standard curves in other food matrices. 100% milk; , solid line; 95% milk, ■, dashed line; egg, ●, dotted line; cantaloupe, ▲, dash-dot line. Insert shows the linear region from 0 to 25 ng/ml. Values are an average of 8 or more squares ± SEM.

Table 1. SEB detection in food matrices		
Matrix	Detection Limit (ng/ml)	Linear Regression Equation (0-25 ng/ml)
Buffer	0.5	y = 1367x + 620
Ham supernatant	0.5	y = 123x + 714
Ham extract	0.5	y = 672x + 1269
Ground beef	0.5	y = 486x - 5
Carcass wash	0.5	y = 305x + 2126
Milk (100%)	0.5	y = 986x - 649
Milk (95%)	0.5	y = 662x – 468
Egg	0.5	y = 736x – 530
Cantaloupe	0.5	y = 541x + 56

ESCHERICHIA COLI O157:H7 DETECTION ASSAY

Based on other reported antibody-based assays for *E. coli* O157:H7 with low levels of detection, the anti-*E. coli* O157:H7 polyclonal antibody from Kirkegaard & Perry Labs, Inc (KPL, Gaithersburg, MD) was biotinylated and used as the capture antibody. Two monoclonal antibodies (Fitzgerald and Biodesign) against *E. coli* O157:H7 and the polyclonal from KPL were labeled with AlexaFluor 647 (AF). Mouse antibodies were harder to label at high d/p ratios without dialysis prior to labeling. Initial studies with these different fluorescently-labeled antibodies were not providing the level of detection required using the typical assay times of 8 minutes for antigen and 4 minutes for reporter antibody. The antigen exposure time was increased to 15 minutes and levels of 10^3 and 10^4 cells/ml were obtained with AF-label KPL polyclonal anti-*E. coli* O157:H7. There was also some problem with the stability of the heat-killed *E. coli* O157:H7 standard from KPL. The signals obtained with freshly reconstituted cells in water were different from those obtained for subsequent days. Reconstituting the cells in glycerol/water

50:50 as suggested by the manufacturer produced more stable results over a week. There is some signal associated with glycerol levels around 7% that are taken into account in determining detection limits. Figure 5 shows a representative image of an anti-*E.coli* slide. In this figure, 10^3 cells/ml is above 3 SEM over blank with 7% glycerol. The studies to generate the standard curve for *E. coli* in buffer are being completed employing biotinylated KPL polyclonal antibody, 15-minute incubation with antigen that was reconstituted with glycerol, and AF-labeled KPL polyclonal antibody.

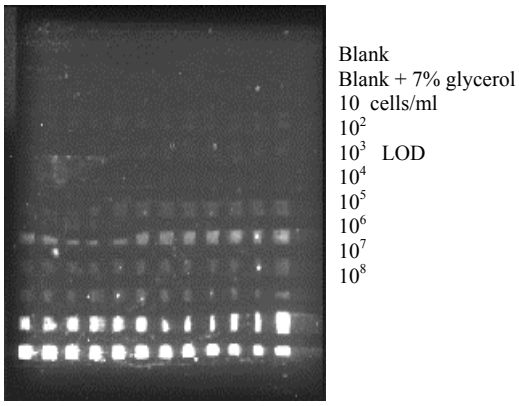


Figure 5: E. coli detection

SALMONELLA DETECTION ASSAY

An assay was developed to detect *Salmonella* in chicken washs and other food matrices. Initial work focused on the selection of the optimum capture/tracer antibody combination for low level detection. The tracer antibodies were labeled with Cy5. The combination of biotinylated Biodesign's rabbit anti-*Salmonella* as the capture antibody and Cy5-labeled monoclonal M32242 from Fitzgerald was selected as the optimum pair. After selection of the antibody combination, a standard curve in buffer was generated. Figure 6 shows the standard curve. The limit of detection was 8 x 10^4, although 3 x 10^4 cfu/ml was observed occasionally.

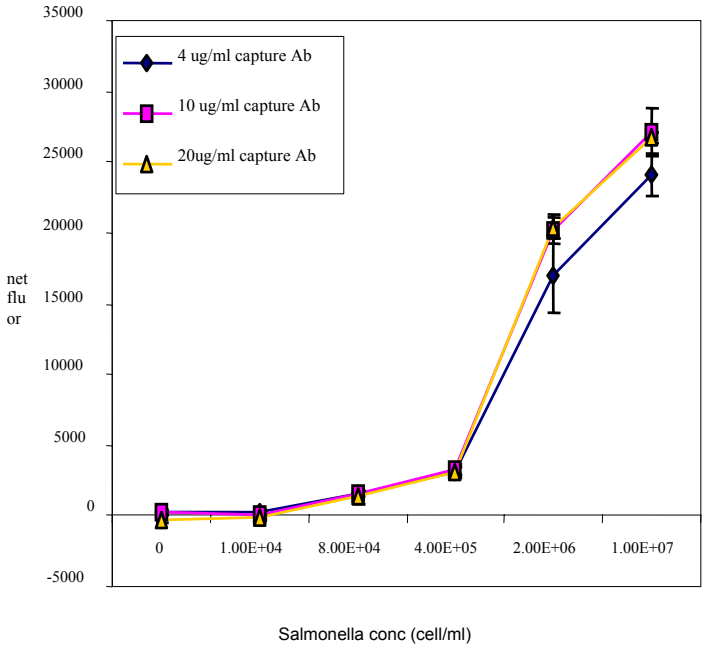
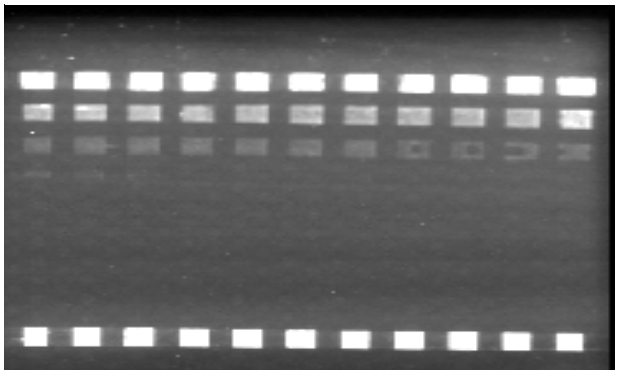


Figure 6: Salmonella

Salmonella was spiked in various food matrices including chicken carcass wash. Figure 7 shows the image of *Salmonella* detection in carcass wash. The bottom row is the positive control in buffer only. Figure 8 shows *Salmonella* standard curves in other food matrices. Another concern is that more than one bacterial agent might be present in a sample. The effect of high levels of two other bacterial agents on *Salmonella* detection was examined. *Salmonella* was still detected at all concentrations in the presence of E. coli and Campylobacter at 10^8 cells per ml.



$1X10^7$ cfu/ml in carcass wash
$2X10^6$ cfu/ml in carcass wash
$4X10^5$ cfu/ml in carcass wash
$8X10^4$ cfu/ml in carcass wash
$1.6X10^4$ cfu/ml in carcass wash
none
buffer only (no Salmonella)
10^7 cfu/ml in buffer (positive control, no food)

Figure 7: Salmonella

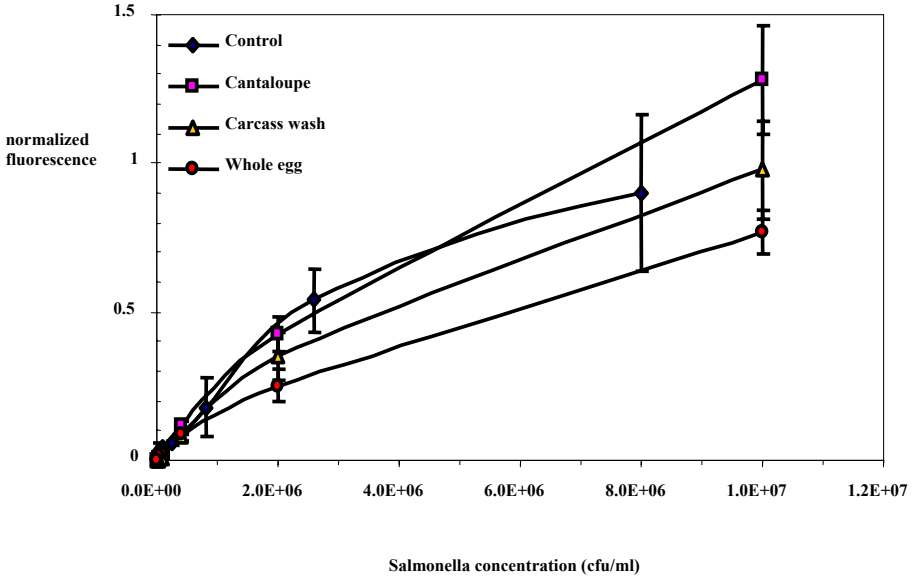


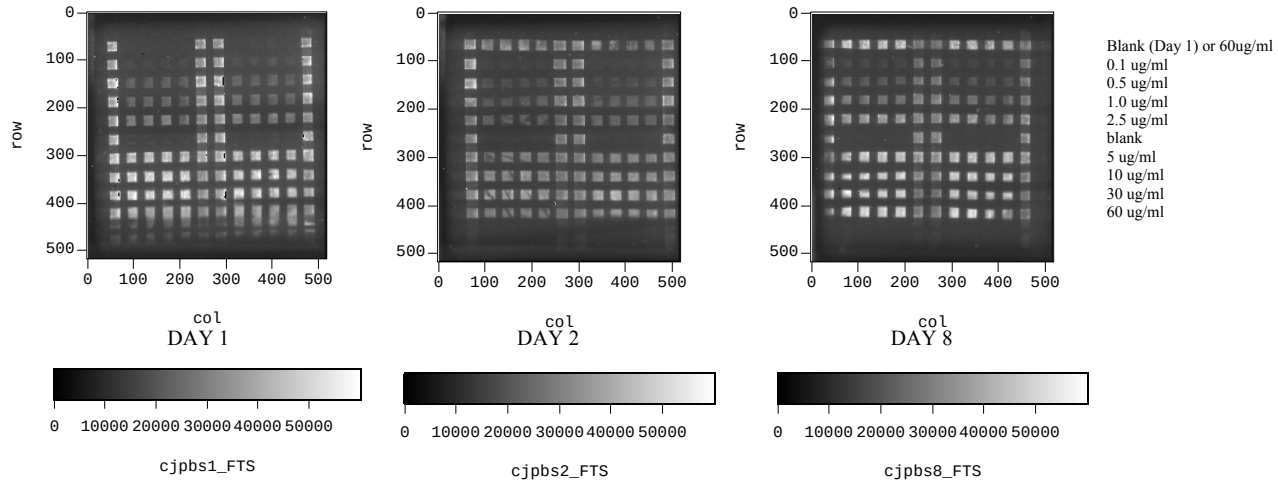
Figure 8: Salmonella

CAMPYLOBACTER DETECTION ASSAY

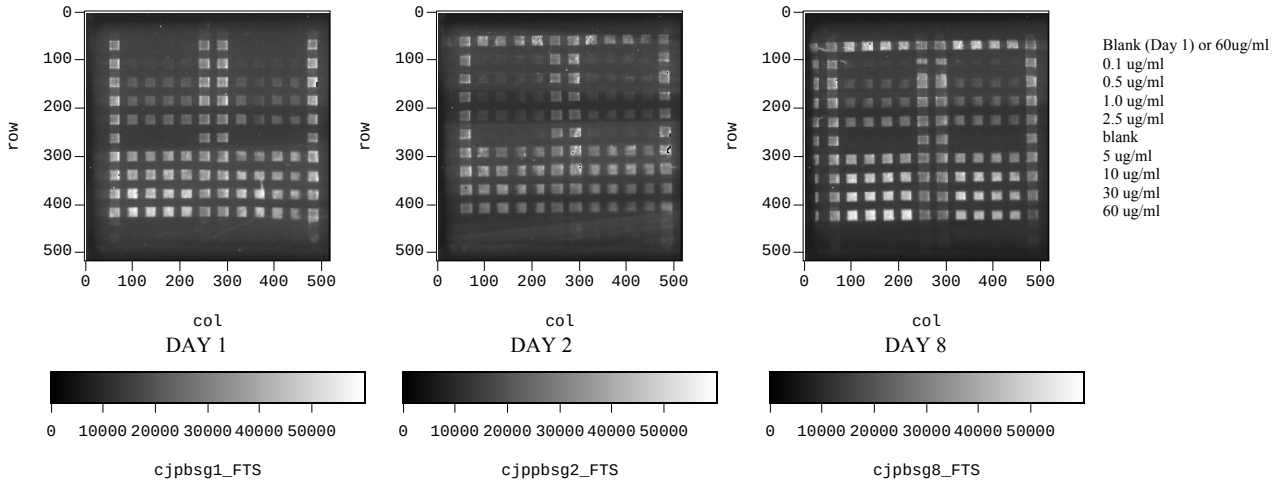
Due to concerns about the stability of *Campylobacter jejuni* solutions prepared and stored prior to use, Time studies were carried out. Fresh *Campylobacter jejuni* solutions were prepared, at each concentration, on day 1 of the study in PBSTB or PBSTB + 10 % glycerol and separated into 3 batches for day 1, day 2 and day 8 of the study. The day 2 and day 8 samples were stored in the refrigerator at + 4°C until required. Anti- Campylobacter was patterned in columns 2-5 and 7-11. As well as the TNT controls (columns 1, 6, 7, 12) run during the assay, a positive control at 60 µg/mL *Campylobacter jejuni*, prepared on the day of the assay, was also run in one of the channels. *Campylobacter jejuni* concentrations are given in µg/mL rather than the standard cfu units more commonly used in publicationsbecause the supplier of the *Campylobacter jejuni*, was unable to provide the equivalent cfu concentration of their product.

From these studies, the antigen *Campylobacter jejuni* can be measured using the rabbit antibodies purchased from Biodesign, with a resulting limit of detection of between 0.1 to 0.5 µg/mL. The time study revealed that storage of the *Campylobacter jejuni* antigen in the refrigerator, even at some of the low concentrations prepared, did not greatly affect the performance of the assay (compare day 1 and day 8). Glycerol was also found to have little effect on the assay performance. (Figures 9 and 10)

(A) *Campylobacter jejuni* stored in PBSTB (Figure 9)



(B) *Campylobacter jejuni* stored in PBSTB + 10 % Glycerol (Figure 10)



Using the same rabbit anti-*C. jeuni* as the capture and the Cy-5 labeled tracer, a standard curve was developed from a single assay. As with the *E. coli* assay, the antigen incubation time was increased to 15 min. It is hard to determine if we are in the appropriate range for detection as all other assays give the units as cfu/ml whereas our units are :g/ml. We are waiting to get standards from another source with information on the cfu's.

LISTERIA DETECTION ASSAY

While we were able to detect 2 :g/ml *Listeria* (in buffer) by ELISA, we have had limited success detecting Listeria using the array sensor. Our current limit of detection for *Listeria* in 15 minute array sensor assays is 20 :g/ml. As our previous source of inactivated *Listeria* antigen was unable to provide us with information regarding the cell content of our *Listeria* standard (i.e., conversion of mg/ml to cfu/ml), we do not currently know how many cells of *Listeria* we are able to detect. We are presently in contact with researchers at FDA to serve as a more reliable source of *Listeria*, *Campylobacter*, and other inactivated stocks.

CONCLUSIONS

Detection of SEB, *Salmonella*, *E. coli* O157:H7, *Campylobacter* and *Listeria* with the Array Biosensor were demonstrated. Levels of detection are similar to or lower than other reported methods while taking less than 30 minutes to perform.. The bacterial assays were performed without the pre-enrichment step used in most protocols. For SEB and *Salmonella*, the biological agents were also tested in several food matrices with little or no loss of detection capability. The ability of the array sensor to test *E. coli* in food samples is underway. Future studies will focus on combining these assays and testing contaminated samples.

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